Chapter 5
An NMR study to help the design of more potent probes

The enol carbamate scaffold contains two putative electrophilic positions, a carbamate and a Michael acceptor. To gain insight into the reactivity of this scaffold we designed an NMR study using an amine, a thiol and an alcohol as nucleophiles and a methyl ester probe bearing the two putative electrophiles. The results obtained showed that thiols react with the Michael acceptor whereas amines and alcohols interact with the carbamate. These findings pushed us to design new probes to improve potency and one of our newly designed sulfone-based probe showed impressive selectivity and potency towards RALDH1 when our acrylate probe did not target this overexpressed protein in A549 lysates. Overall, the results obtained in this chapter show the versatility of the enol carbamate scaffold and its potential for application in the field of activity-based probes.

5.1 Tweaking original electrophiles

The natural product Brabantamide A has antibacterial, antifungal and antioomycete activity and it has therefore been employed as an inspiration for the development of enolcarbamate inhibitors for Lp-PLA$_2$, penicillin-binding proteins (PBPs) and *B.subtilis* esterase.[1-3] Using mass spectrometry, Thirkettle *et al.*[4] showed that Brabantamide A carbamoylates Lp-PLA$_2$ and based on the fluorescent labeling of Lp-PLA$_2$ and *B. subtilis* esterase with the enolcarbamate probes described in Chapter 4, it is safe to conclude that this scaffold covalently modifies serine hydrolases in general.[3] Furthermore, we demonstrated in Chapter 3 that bicyclic enolcarbamates also covalently modify hyperreactive cysteine residues in aldehyde dehydrogenases. These biological activities make enolcarbamates attractive leads for the synthesis of ABPs and inhibitors, but to generate selectivity both the affinity and reactivity needs to be optimized concomitantly. Finding the optimal balance between reactivity (i.e. potency) and selectivity is essential to modify proteins of interest in biologically relevant samples. Improving the affinity generally requires understanding of the binding pockets of the target proteins. By studying the interactions made in these pockets, the substitution pattern can be adjusted to optimize binding for the respective protein. For the reactive groups, it has been shown that increasing the electrophilic character, for example by introducing groups that are more electron withdrawing, generally improves the inhibitory potency. However, overdoing this causes a corollary loss of selectivity. Electrophiles that are too reactive modify any (good) nucleophile in the proximity. As a result, the ABPs and inhibitors containing these electrophiles will be less specific and potentially display off-target toxicity. Furthermore, it has been shown that the selectivity of probes and inhibitors can be enhanced by decreasing the electrophilicity, but this often comes at the expense of a reduced potency.

Under the light of these considerations, it is evident that tuning the reactivity of an electrophile requires understanding of the mechanism by which the target proteins are modified. For enol carbamates, this is not fully understood but they share structural similarities with both activated carbamates and Michael acceptors. Activated carbamates have been shown to inhibit rather selectively serine hydrolases[5] by carbamoylating the catalytic serine residue in a mechanism-based fashion.[6,7] As for the natural substrate, carbamate inhibitors are attacked by the active site serine forming a tetrahedral intermediate. After expelling the alcohol leaving group, a

carbamate enzyme adduct is produced, which is similar to substrate-enzyme intermediate formed during catalysis. The latter rapidly hydrolyzes, but delocalization considerably slows down hydrolysis of the carbamate adduct. Since the carbamate within the inhibitor also slows down the initial step (i.e. attack by the catalytic serine), a good leaving group is required to increase the efficiency of the first step and thus the efficiency of enzyme modification.[8] Brabantamide A derivatives have, similar to reported activated carbamates, a good leaving group since the pKa of an enol amide is relatively low (the enol amide is not a leaving-group per se as it would stay attached to the enzyme-inhibitor adduct after the reaction) (Figure 1C). In addition to this, the reactivity of enolcarbamates is further increased by the nitrogen being at a bridge-head position (Figure 1A), leading to a twisted amide therefore out of conjugation (disfavored delocalization according to Bredt's rule[9,10]). Therefore, enolcarbamates should undergo nucleophilic attack by the serine residue readily (Figure 1B).

Michael acceptors undergo conjugate addition with cysteine residue.[5] Nucleophilic attack by a thiol on the β-carbon of an α,β-unsaturated carbonyl leads to a negatively charged enolate, which is rapidly protonated finally resulting in the covalently modified Michael adduct. It is well known that α,β-unsaturated carbonyls react with thiols in biological samples.[11] The α,β-unsaturated amide in the brabantamide scaffold can therefore also potentially function as a Michael acceptor (Figure 1A), in particular because retro-Michael reaction followed by CO_2 extrusion should be a considerable driving force (Figure 1D).

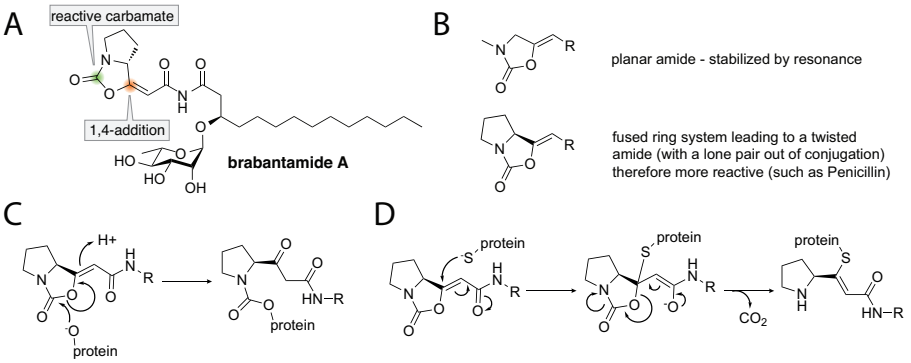


Figure 1. Brabantamide scaffold (A); in a planar amide the C-N bound has a significant double bound character making them more difficult to cleave than twisted amides (B); carbamate react with an alcohol (C) or the Michael acceptor react with a thiol (D).

Based on the structural resemblance with both activated carbamates and Michael acceptors, we hypothesized that nucleophilic serine and cysteine residues react with the electrophilic carbamate and the α,β-unsaturated amide respectively. To determine if this is indeed the case, we performed in this Chapter model studies on the enol carbamate scaffold. Methyl ester **2** (Figure 4) was reacted with alcohols, amines and thiols and the products were analyzed by NMR. These experiments highlight the selectivity of both electrophilic traps towards nucleophiles. We subsequently aimed to tune the reactivity of towards serine and cysteine residues. To decrease the reactivity, we prepared monocyclic carbamate derivatives (Figure 3B) and to increase the reactivity we synthesized α,β-unsaturated sulfone or ester derivatives of probe **PJD2d** (Figure 3A and 3B)(Figure 2). We studied the reactivity of these derivatives using activity-based protein profiling on recombinant mammalian Lp-PLA2, esterase from *Bacillus subtilis* and mammalian RALDH1 and in lysates from *Bacillus subtilis* and mammalian cell line A549.

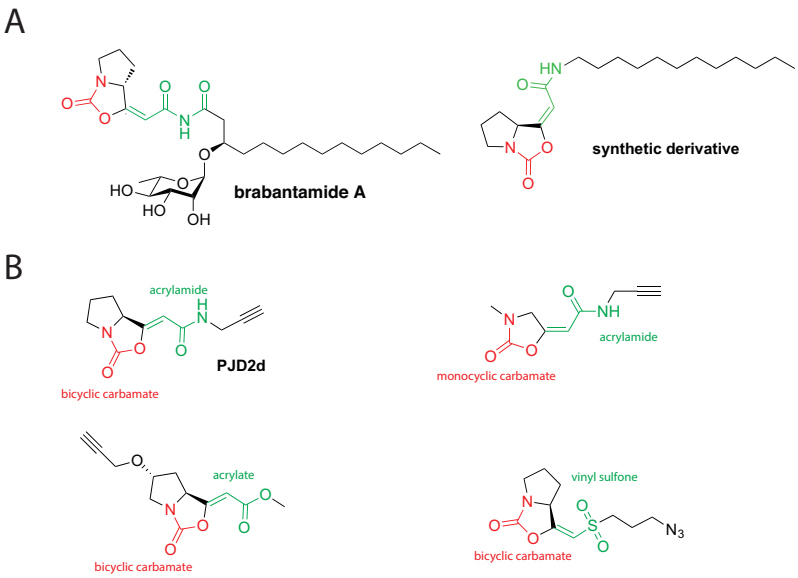


Figure 3. Structure of natural product brabantamide A and synthetic derivative prepared by Pinto et al.[2] (A); structure of brabantamide inspired scaffold **PJD2d** (Chapter 2 and 3) and fine-tuned derivatives (B). Carbamates and Michael acceptors are highlighted in red and green respectively.

5.2 Mechanistic studies

To study the chemical reactivity of enol cyclocarbamates, we prepared known methyl ester **2** from readily available β-keto ester **1** using the deprotection-cyclization protocol described in Chapter 2. Methyl ester **2** was obtained as a mixture of *E* and *Z* isomers in 56 % yield, with the latter being the major isomer. The chemical shifts of the isomers correspond with those reported in literature (Figure 4).[3,12]

Once we had model compound **2** in hand, we examined its reactivity with alcohols, amines and thiols. We first determined if the *Z*-isomer of **2** reacted with alcohols by dissolving **2** in anhydrous methanol containing 1 equivalent of K_2CO_3. Under these conditions, methanol reacted quickly with the *Z*-isomer of **2**. NMR analysis of the obtained product **3** gave ^{1}H-NMR intermediate **1** (Figure 5).

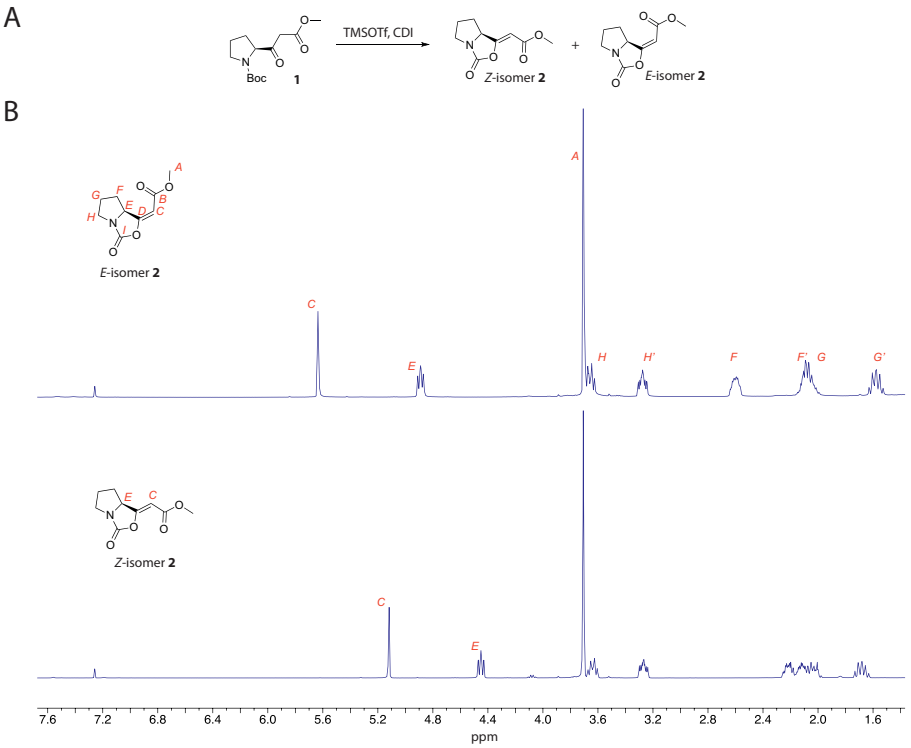


Figure 4. Synthesis of model compound **2**. (A); ^{1}H-NMR of *Z* and *E* isomers of enol carbamate **2** (B). Typical chemical shifts (based on literature and NOESY experiment performed in Chapter 2) of the double bond proton and the α-proton are highlighted with the C and E respectively.

The ^{13}C-NMR of **3** contained peaks at 203.4 and and ^{13}C-NMR spectra that are very similar to Boc-protected β-keto ester 203.1 ppm, which are indicative for the presence of the ketone. Furthermore, the characteristic peaks for the carbamate were observed at 155.9 and 154.9 ppm (Figure 5A). As for **1**, the product of the reaction with methanol gave a mixture of rotamers in the NMR, which is typical for carbamate protected-proline derivatives. The HRMS supported that methyl carbamate **3** had formed and we therefore concluded that methanol reacted with the carbamate within **2**, resulting in ring opening.

The reactivity with thiols was also examined. *Z*-isomer **2** dissolved in acetonitrile was added to an alkaline solution containing 1 equivalent of thiophenol. After full consumption of the starting material, the reaction mixture was extracted with diethyl ether. NMR analysis of the extracted products revealed that the double bond isomerized to the *E*-isomer under

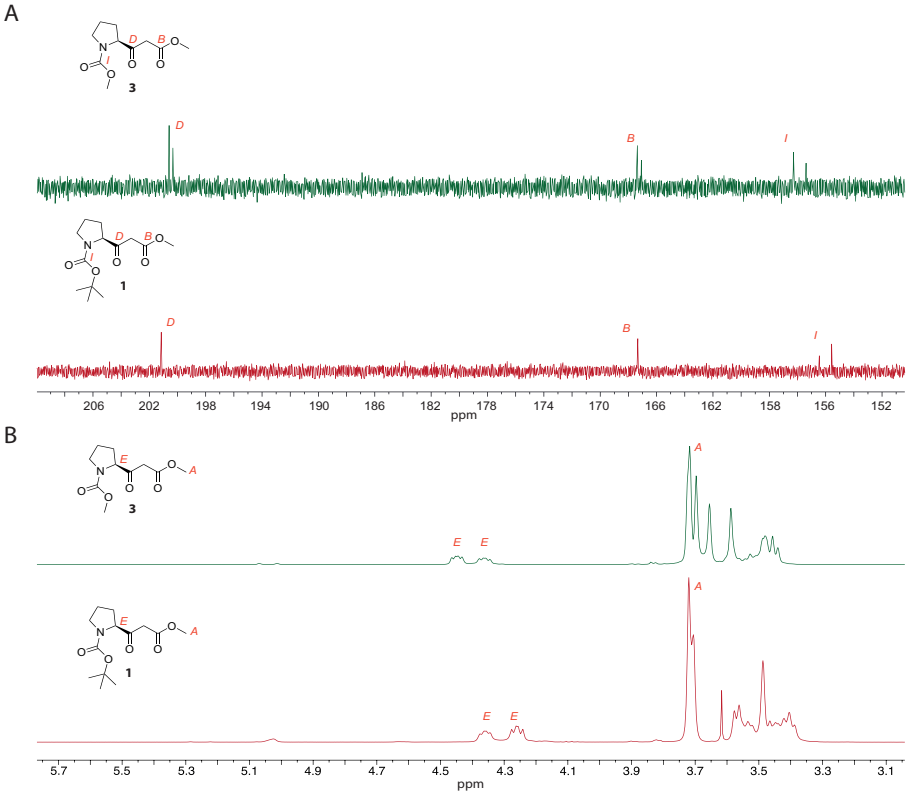


Figure 5. (A) ^{13}C-NMR of product **3** (top) and closely related intermediate **1** (bottom); (B) ^{1}H-NMR of product **3** (top) and closely related intermediate **1** (bottom).

these reaction conditions. Isomerization presumably occurs via 1,4-addition followed by a retro Michael reaction with extrusion of thiophenol (Figure 6B). By TLC, we observed that a product remained in the aqueous layer and we therefore neutralized the solution with H_2SO_4 and extracted it with diethyl ether. The product obtained from the neutralized layer contained characteristic peaks around 6.5 ppm in the ^{1}H-NMR and 111.56 ppm in the ^{13}C-NMR. These chemical shifts suggest the formation of thioenol ether **4** and HRMS analysis proved that this product was obtained. This product is presumably formed by conjugate addition of thiophenol and a subsequent retro Michael reaction (Figure 6C). To verify these results, we followed the reaction between thiophenol and enol cyclocarbamate **2** by NMR. Mixing thiophenol and **2** in $CDCl_3$ did not result in a reaction according to NMR (Figure 7A and 7B). We then added triethylamine (Et_3N), which immediately promoted the reaction.

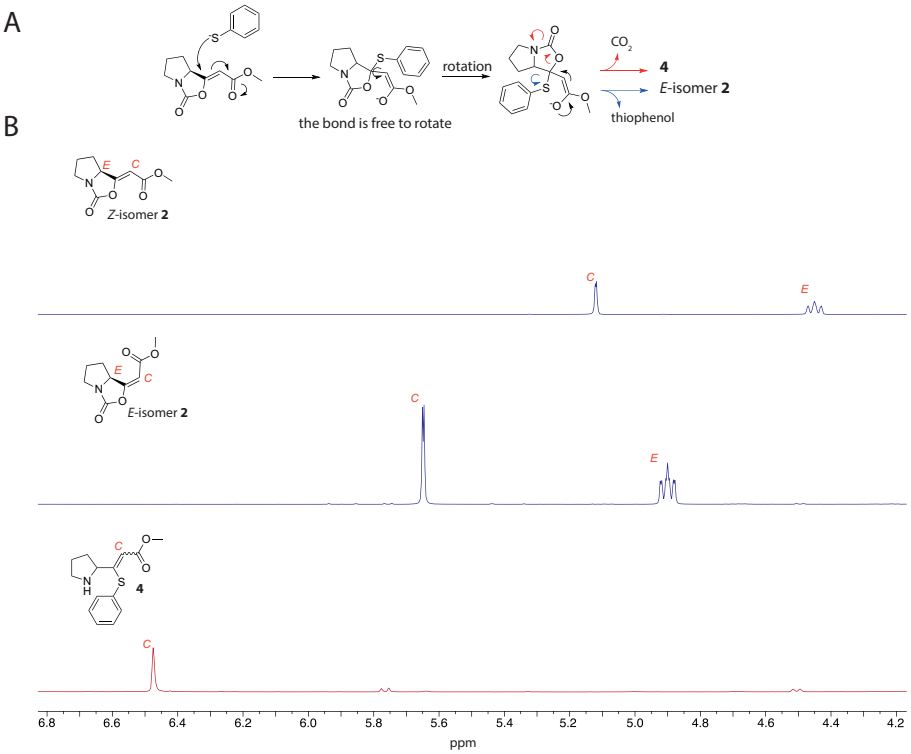


Figure 6. (A) Mechanism leading to compound **4** and *E*-isomer **2**. (B) ^{1}H-NMR of starting material *Z*-isomer **2** (top panel), *E*-isomer **2** obtained after isomerization (middle panel) and the thiophenol adduct **4** (bottom panel).

The typical double bond signal of the enol carbamate decreased and a new peak appeared at 5.9 ppm (Figure 7C), which possibly belongs to one of the isomers of the thioenol ether **4** or the thioketal intermediate. Upon silica gel flash column chromatography, this product converted into the same product **4** as that from the reaction in acetonitrile/water (Figure 7D).

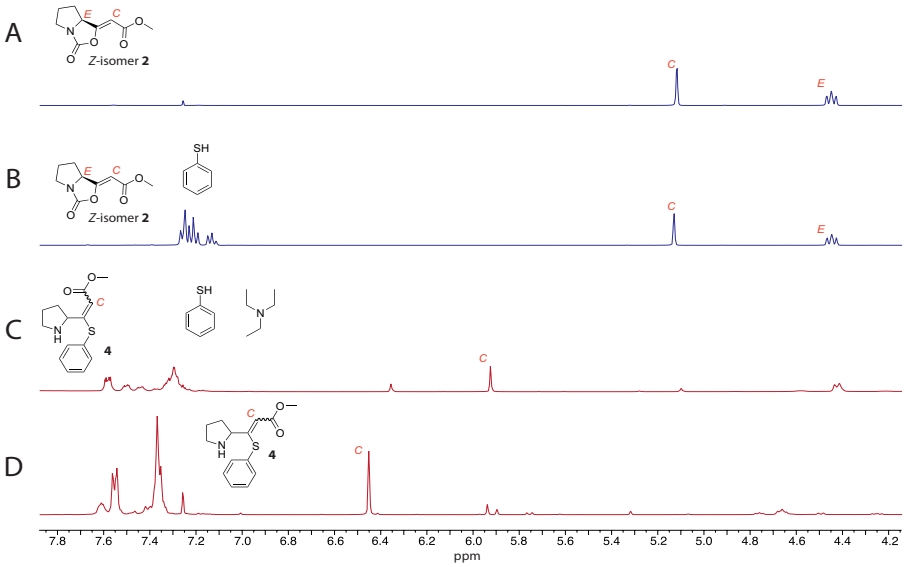


Figure 7. ^{1}H-NMR of starting material Z-isomer **2** (A), unreacted starting material **2** and thiophenol (B) intermediate after addition of Et_3N (C) and the thiophenol purified adduct **4** (D).

Finally, we examined if enol cyclocarbamate **2** also reacted with amines. To the enol cyclocarbamate in $CDCl_3$ was added aniline. After overnight incubation and heating to 40 °C, no conversion was observed by ^{1}H-NMR (Figure 8B). We then added the more reactive benzylamine, which slowly reacted with the enol cyclocarbamate (Figure 8C and 8D). As judged by NMR, the starting material disappeared over the course of the reaction and a new compound formed. This product showed specific peaks of the benzylamine urea adduct **5**, namely a ketone peak at 203 ppm in ^{13}C NMR and the typical *NH* and the α-proton labeled *E* in ^{1}H NMR, see Figure 8D. Interestingly, upon purification of the product by silica gel chromatography, the ketone peak disappeared and a new peak at 86 ppm in ^{13}C NMR and at 4.99 ppm in ^{1}H-NMR appeared. Similar shifts have been reported for cyclized urea adducts, suggesting that we obtained the bicyclic benzylamine adduct **6** (Figure 8E). HRMS proved that bicyclic adduct **6** had formed. The

acidic silica apparently catalyzed ring closure during purification. As hypothesized, both the electrophilic traps found in enol cyclocarbamate **2** react with specific nucleophiles.

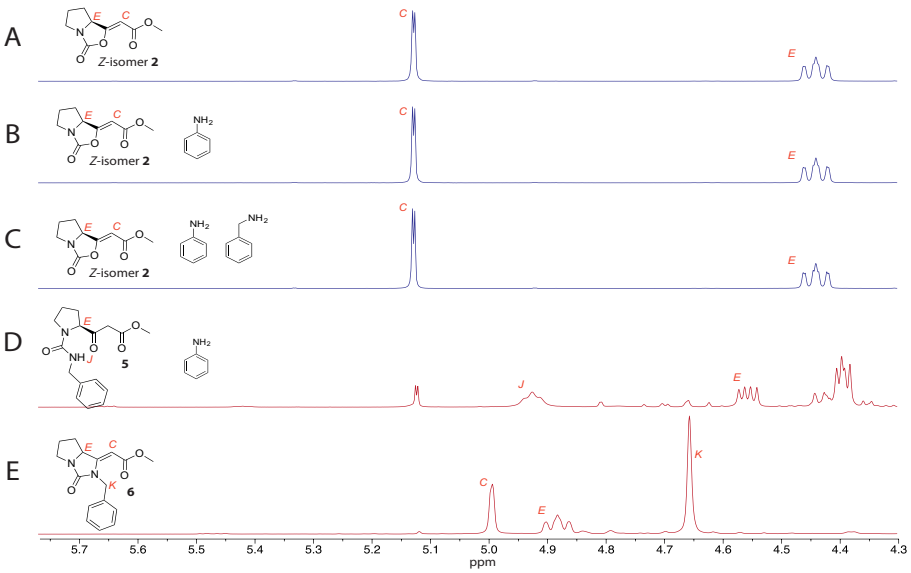


Figure 8. ^{1}H-NMR of starting material *Z*-isomer **A** (A); unreacted starting material **A** and aniline (B); unreacted starting material **A**, aniline and benzylamine (right after addition of the latter) (C); intermediate open adduct **5** (D); closed adduct **6** obtained after purification (E).

The carbamate within this scaffold undergoes 1,2-addition by amines and alcohols leading to carbamoyl adducts. The products from amine addition react further during purification over silica to the ring closed products. Thiols are alkylated by conjugate addition to the α,β-unsaturated ester. The subsequent retro-Michael reaction either leads to isomerization of the enol carbamate *via* thiophenol extrusion or to the decarboxylated thiophenol adduct *via* CO_2 extrusion.

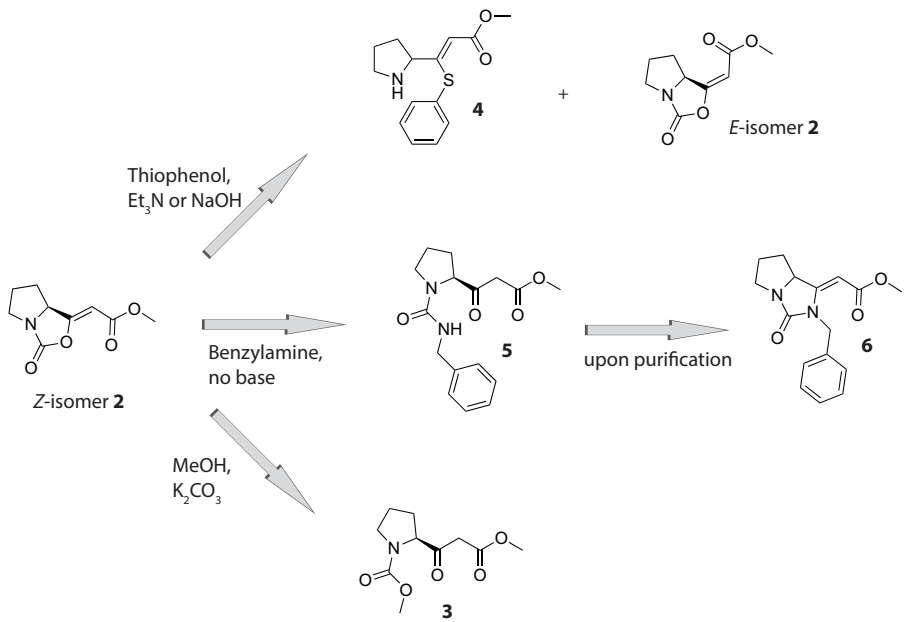


Figure 9. Overview of reactivities of enol-cyclocarbamate **2**.

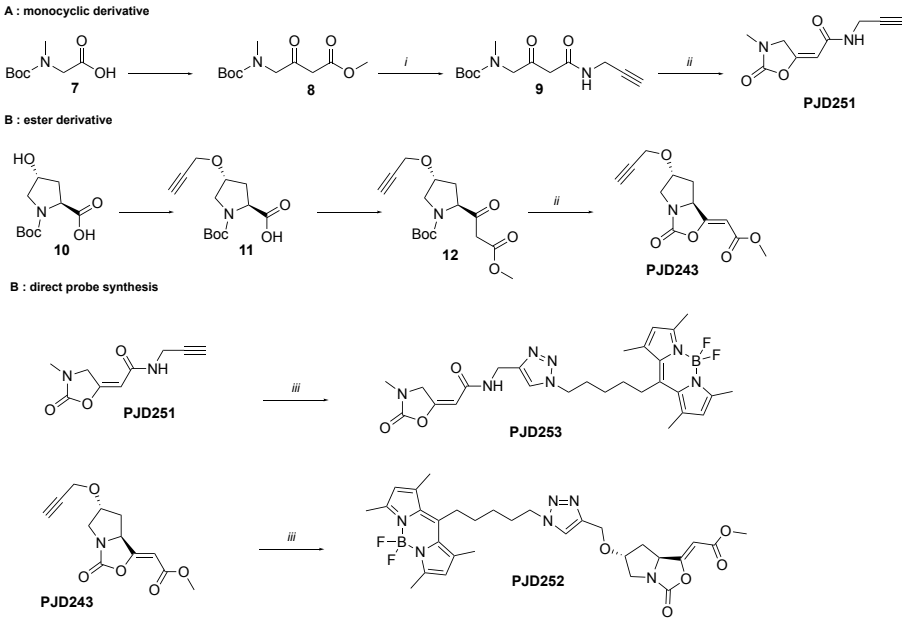
5.3 Tuning the reactivity of the enol carbamates. Synthesis and biocharacterization

The mechanistic studies unambiguously demonstrate that both electrophilic centers react with a specific subset of nucleophiles commonly found in Nature. We therefore aimed to alter the scaffold to tune the reactivity towards a specific class and/or to increase the general potency of the compounds using the reported selectivity studies on activated carbamates and Michael acceptors as guidelines. For activated carbamates, it has been shown that the leaving group has a large effect on the reactivity and selectivity. The pKa of the leaving group determines to a large extend which serine hydrolases are labeled and by varying the leaving group (phenols, hexafluoroisopropanol (HFIP), *O*-*N*-hydroxysuccinimide (NHS) and *N*-hydroxyhydantoins) selectivity has been obtained.[13] Also the part that remains on the protein has been varied to target serine hydrolases of interest via non-covalent interaction. The reactivity of Michael acceptor has also been tuned in order to increase or decrease the electrophilic character. The more electron-deficient the C-C double bond is, the more susceptible it is to Michael addition and consequently, the order of reactivity is as follows, maleimide > vinyl sulfone > acrylate > acrylamide (Figure 2B).[14,15]

Based on this, we hypothesized that replacing the β-ketoamide motive with β-ketosulfone and β-keto esters would both make the CC double bond more electron deficient and increase the reactivity of the carbamate. The alpha protons of these groups are considerably more acidic than those of a β-ketoamide.[14] As such, these modifications should increase the reactivity in general. Furthermore, we reasoned that the reactivity of the carbamate could be reduced by synthesizing monocylic analogues. Finally, the substitution pattern on the scaffold can affect the selectivity of the probe. In chapter 3 and 4, we already demonstrated that modifications at the bicyclic head-group impair labeling of RALDH1, but did not seem to have an effect on the serine hydrolase inhibition. We therefore incorporated the tag at different positions and studied the effect on the selectivity.

5.3.1 Chemical synthesis

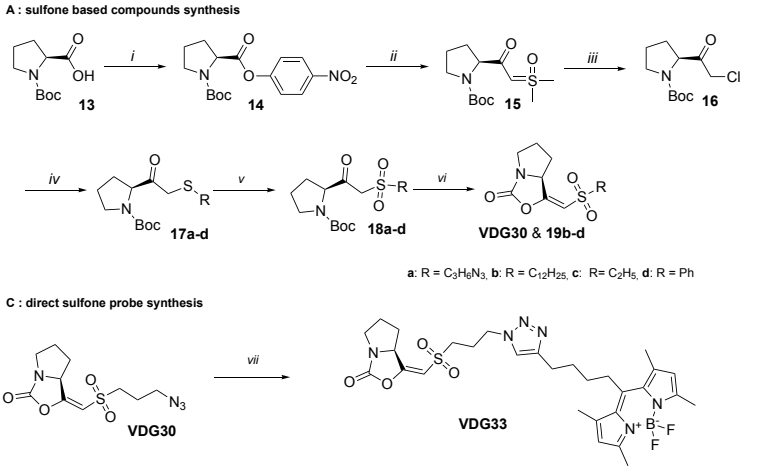
The monocylic compound **PJD251** was obtained from sarcosine derived β-keto ester **8** for which the synthesis is described in Chapter 2. β-keto ester **8** was reacted with propargyl amine under the agency of DABAL-Me_3 to form



Scheme 1. Synthesis of monocyclic enol carbamate **PJD251** and ester derivative **PJD243**. Reagents and conditions: (i) DABCO, $AlMe_3$, propargyl amine; yield: 55%; (ii) TMSOTf then CDI (yield over 2 steps: 10–20%), (iii) $CuSO_4$, sodium ascorbate, BODIPY-N_3 (23-48%).

β-keto amide **9**. Subsequent removal of the Boc group with TMSOTf in CH_2Cl_2 and immediate cyclization with 1,1'-carbonyldiimidazole (CDI) gave monocyclic probe **PJD251** (Scheme 1). Ester-based probe **PJD243** was prepared in analogous manner from β-keto ester **12** (for synthesis of **12**, see Chapter 2). Deprotection of the amine followed by cyclization with CDI afforded probe **PJD243** in 55 % yield. Both **PJD243** and **PJD251** were subjected to copper-catalyzed cycloaddition to implement the fluorescent BODIPY to respectively obtain **PJD252** and **PJD253**.

To synthesize vinyl sulfone probes **VDG30** and **VDG33**, and inhibitors **19b-d**, we devised a novel route. We reasoned that all of the sulfone probes could be obtained from *N*-Boc-L-proline-α-chloroketone **16**, as this key intermediate is readily modified with different thiol containing reagents (Scheme 2). Therefore, chloroketone **16** was prepared from *N*-Boc-L-proline-4-nitrophenol ester **14** using an adapted procedure of Wang *et al.*[16]. Activated ester **14** was reacted with dimethylsulfoxonium methylide generated *in situ* by treating trimethylsulfoxonium iodide with potassium *tert*-butoxide.



Scheme 2. Synthesis of vinyl sulfone based compounds. Reagents and conditions: (i) *p*-nitrophenol, DCC, DMAP modified procedure from Kovacs *et al.*[17]; (ii) potassium *tert*-butoxide, trimethylsulfoxonium iodide; (iii) LiCl, camphorsulfonic acid; (iv) *S*-(3-azidopropyl)thioacetate, K_2CO_3 for **17a**, 1-dodecanethiol for **17b**, thiophenol for **17c**, ethanethiol for **17d**; (v) *m*CPBA ; (vi) TMSOTf, CDI; (vii) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, BODIPY-alkyne.

The obtained ylide **15** was then converted into chloroketone **16** using lithium chloride and camphorsulfonic acid as a dry HCl source. In our hands, methanesulfonic acid did not afford the desired chloromethyl ketone, presumably due to the presence of water. Key intermediate chloromethyl ketone **16** was subsequently used to prepare **VDG30** and **19b-d**. Hydrolysis of S-(3-azidopropyl)thioacetate with K_2CO_3 in methanol/water in the presence of chloroketone **16** gave *N*-Boc-L-proline-(3-azidopropyl)sulfide **17a** in good yields. Reacting **16** with 1-dodecanethiol, thiophenol and ethanethiol afforded thioethers **17b-d**. Oxidation of sulfides **17a-d** with *meta*-chloroperoxybenzoic acid (*m*CPBA) efficiently yielded the corresponding sulfones **18a-d**. As a final step to synthesize enol cyclocarbamate **VDG30** and **19b-d**, we used our previously reported one-pot deprotection-cyclization step. Reacting the Boc-protected β-ketosulfones with TMSOTf for 4 h followed by the addition of CDI gave *E*-isomer of the desired sulfone derivatives in good yields. The two-step probe **VDG30** bearing an azide was then further functionalized by a copper-catalyzed click reaction to incorporate the fluorescent BODIPY in order to obtain direct probe **VDG33**.

5.3.2 Activity-based protein profiling

The biological activity of the direct probes **VDG33**, **PJD252** and **PJD253** was first studied on the serine hydrolases PLA2G7 and *B.subtilis* esterase and the dehydrogenase RALDH1. To this end, the purified enzymes were incubated with the probe and the labeling intensity was compared to **PJD224**. By implementing a sulfone instead of the amide as an electron-withdrawing group, we were expecting to increase the reactivity of the corresponding Michael acceptor. Indeed we observed a 2 fold more intense fluorescent signal for RALDH1 treated with **VDG33** compared to **PJD224** (at 1 μM). Incubating the esterase of *B.subtilis* with the same probes revealed that both probes labeled this serine hydrolase with equal efficiency. Finally, the fluorescence intensity of Lp-PLA_2 reacted with the sulfone probe **VDG33** is slightly more intense than **PJD224** at 0.1 μM, which suggest that this enzyme also reacts more efficiently with the sulfone probe. The direct probes **PJD252** and **PJD253** were tested on the same proteins. Interestingly, monocyclic probe **PJD253** displayed weak labeling and did not label any of the proteins (or very weakly). On the contrary ester probe **PJD252** labeled efficiently RALDH1 and the esterase from *B.subtilis* but did not label Lp-PLA_2 at the tested concentrations (Figure 10).

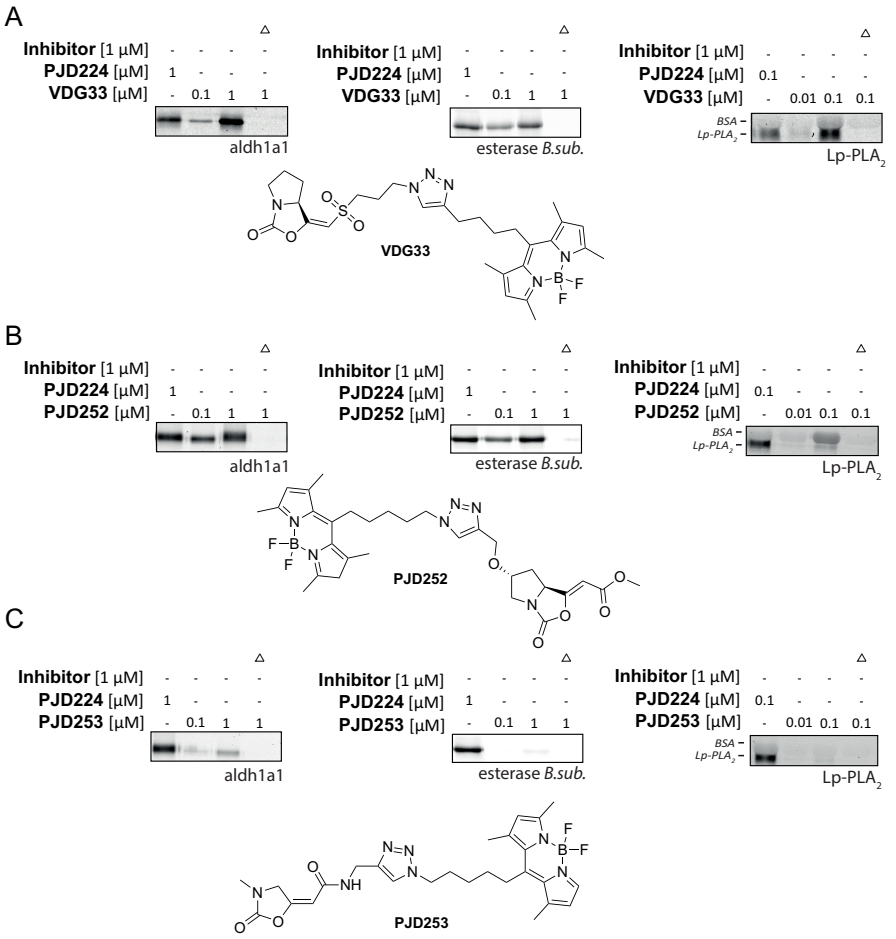


Figure 10. Initial characterization of probes **VDG033** (A), **PJD252** (B) and **PJD253** (C) on recombinant RALDH1, esterase from B.subtilis and Lp-PLA2 compared with **PJD224**.

These promising initial results on recombinant proteins prompted us to test these compounds in lysates from A549 cells and *Bacillus subtilis*. We assessed the reactivity and selectivity of the new probes by comparing their labeling profile to those of reference compounds **PJD2d** and **PJD224** and by competing them with broad-spectrum serine hydrolase probe Fp-rhodamine. First, lysate of A549 was incubated with increasing concentrations of two-step probes **PJD2d**, **PJD243**, **PJD251** and **VDG30** for one hour and subsequently the lysate was treated with Fp-rhodamine to label remaining serine hydrolase activity. The proteins that reacted with the

two-step probes were visualized with either BODIPY-azide or BODIPY-alkyne using copper catalyzed azide alkyne cycloaddition. Ester derivative **PJD243** and monocyclic analogue **PJD251** did not show very intense labeling at 1 and 10 μM (Figure 11). However, vinyl sulfone **VDG30** labeled the same target protein as reference compound **PJD2d**. Labeling of RALDH1 by **VDG30** is significantly stronger than **PJD2d** (Figure 11). Besides RALDH1, several less abundant proteins seem to be labeled that could belong to the serine hydrolase family. The output in the Cy3 channel confirms that **VDG30** and **PJD243** inhibit labeling of LyPLA1 and LyPLA2 at the used concentrations. A similar pattern was observed for the direct probes **PJD252** and **VDG33**. **VDG33** prominently labeled RALDH1 at 10 and 100 μM (Figure 11). Lowering the probe concentrations (0.01, 0.1 and 1 μM) led to a reduction in the probe signal, but at the same time reduced labeling of serine hydrolases, as judged from the BODIPY and the Fp-rhodamine signal. Interestingly, while **PJD224** targets both ALDH3A1 and RALDH1 at 1 μM, **VDG33** solely reacted with RALDH1 at the same concentration. Up to 10 μM, **VDG33** remains more selective and potent towards RALDH1 than **PJD224**.

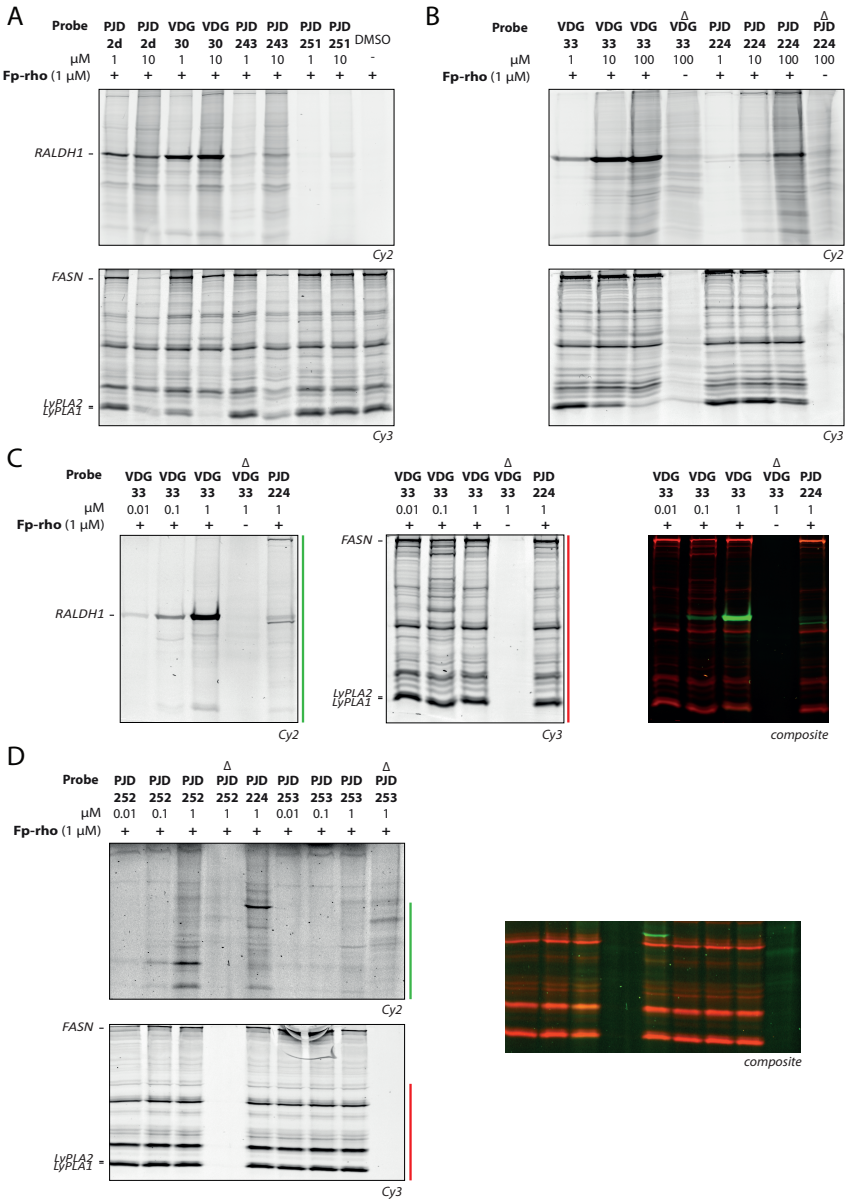


Figure 11. A549 lysates labeling. Labeling profile of two-step probes **PJD2d**, **VDG30**, **PJD243** and **PJD251** (A) and direct probes **VDG33** and **PJD224** (B); labeling profile of direct probes **VDG33** and **PJD224** (Cy2, green) vs Fp-rhodamine (Cy3, red) (C); labeling profile of direct probes **PJD252**, **PJD253** and **PJD224** (Cy2, green) vs Fp-rhodamine (Cy3, red) (D).

Competition experiments between **VDG33** and an excess of iodoacetamide, disulfiram or **PJD2d** confirm that the probe modifies a hyper reactive cysteine residue and that the target is indeed RALDH1 (Figure 12).

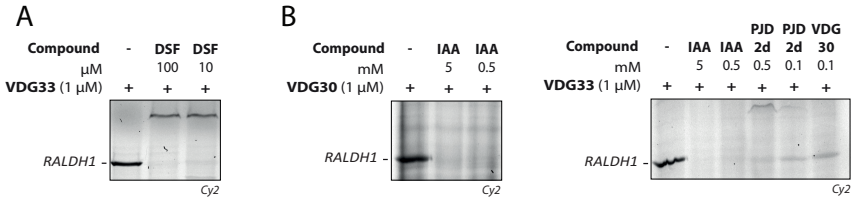


Figure 12. A549 lysates labeling. Competition with Disulfiram (DSF) and the sulfone based probes **VDG33** (A) and competition between iodoacetamide (IAA) and **VDG30** and between IAA, **PJD2d** or **VDG30** and direct probe **VDG33** (B).

As expected from the results obtained with the recombinant proteins and the results with the two-step probes on cell lysates, the monocylic probe **PJD253** displayed poor reactivity on A549. Interestingly, the ester-based probe **PJD252** selectively target two proteins on A549 lysates that we could identify as serine hydrolases by preparing the overlay with the Fp-rhodamine profile (Figure 11).

The same experiments were performed on lysate from *B.subtilis.* Two-step probes **PJD2d** and **VDG30** label a similar subset of proteins and no difference in selectivity or potency was observed (Figure 13). The Fp-rhodamine competition revealed that **PJD2d** and **VDG30** inhibit several proteins around 20-30 kDa. As for A549 lysates, ester derivative **PJD243** and monocyclic analogue **PJD251** did not show very intense labeling at 1 and 10 µM on *B.subtilis* (Figure 13A). Treating *B.subtilis* with 1 µM of monocyclic probe **PJD253** did not result in prominent labeling. Using ester probe **PJD252**, vinyl sulfone probe **VDG33** and amide probe **PJD224** resulted in similar profiles (Figure 13D and 13B). However, at low concentrations vinyl sulfone **VDG33** displayed a remarkable selectivity towards a protein of around 20-30 kDa.

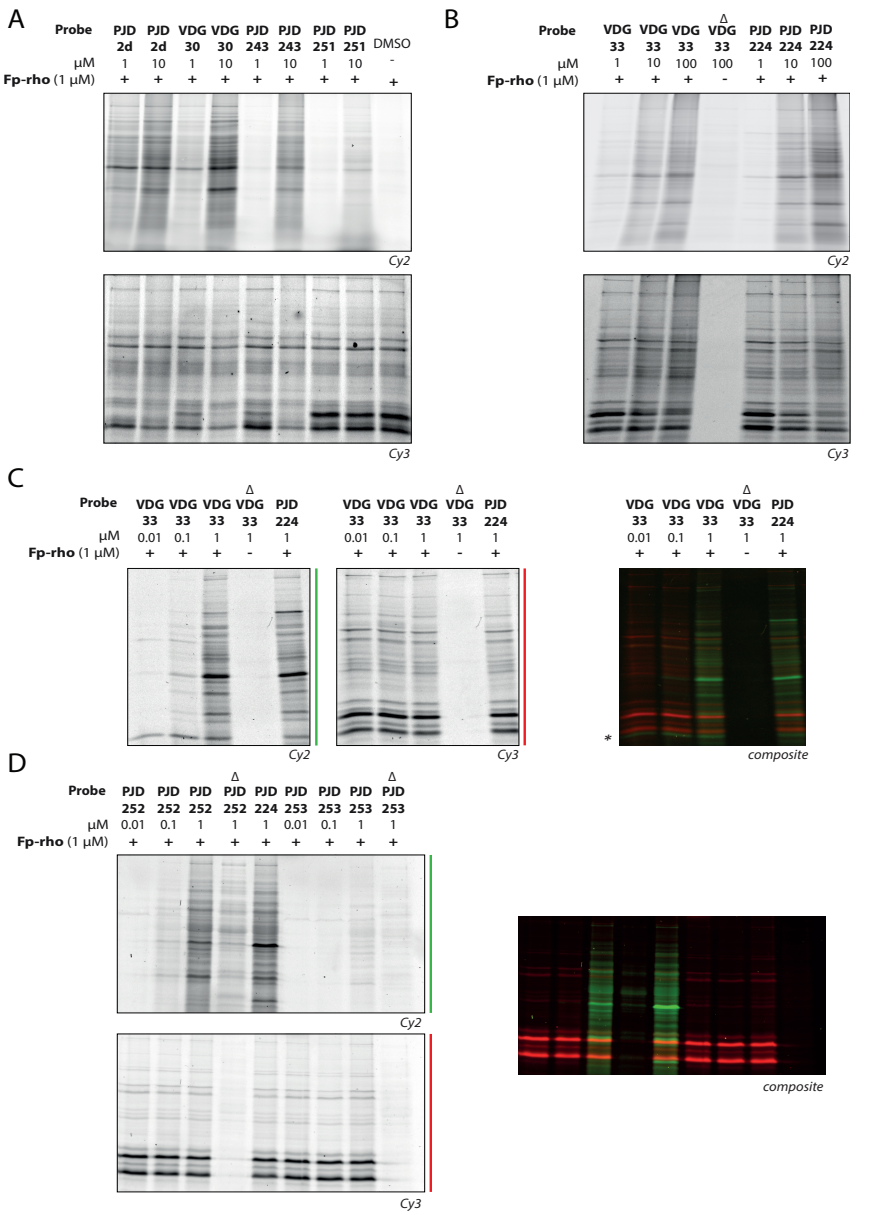


Figure 13. *Bacillus subtilis* lysates labeling. Labeling profile of two-step probes **PJD2d**, **VDG30**, **PJD243** and **PJD251** (A) and direct probes **VDG33** and **PJD224** (B); labeling profile of direct probes **VDG33** and **PJD224** (Cy2, green) vs Fp-rhodamine (Cy3, red) (C); labeling profile of direct probes **PJD252**, **PJD253** and **PJD224** (Cy2, green) vs Fp-rhodamine (Cy3, red) (D).

The overlay of the fluorescence signals detected in the Cy2 and TAMRA channels, which correspond to the proteins that reacted with the probe and proteins that reacted with Fp-rhodamine respectively, suggest that the specific protein labeled by **VDG33** is not a serine hydrolase (Figure 13C). Unfortunately, the two-step probe **VDG30** did not label this protein, which left us without any means to identify this protein and confirm this hypothesis.

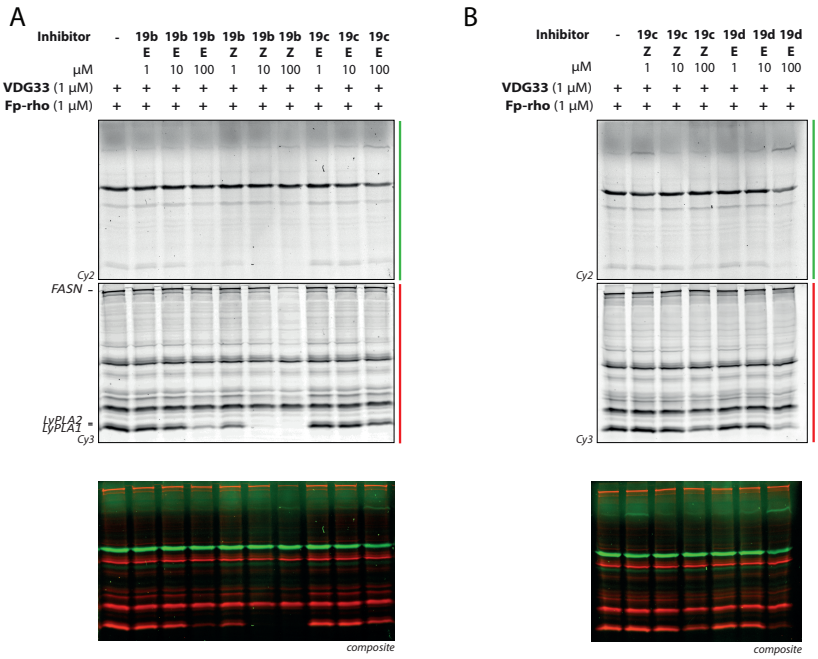


Figure 14. A549 lysates labeling. Screening of sulfone based inhibitors **19b-*E*** and ***Z***, **19c-*E*** (A) and **19c-*Z*** and **19d-*E*** (B) *vs* **VDG33** and Fp-rhodamine on A549 lysates.

We then decided to use the platform offered by **VDG33** to screen inhibitors against RALDH1 together with Fp-rhodamine to assess the potency of the sulfone based inhibitors **19b-d**. We could observe a slight competition at 100 µM with compounds **19c-E** and **19d-E** towards RALDH1 together with a competition on LyPLA1 and LyPLA2. The other compounds did not seem to compete for labeling with RALDH1 even though compounds **19b-E** and **19b-Z** did target LyPLA1 and LyPLA2 at 10 and 100 µM.

The labeling profiles obtained for the newly synthesized sulfone, ester and monocyclic probes are extremely intriguing as it seems that the specificity towards one or another family of proteins (or nucleophiles) can be obtained

if the reactivity of the corresponding electrophiles is carefully optimized. These findings should guide further development and applications of this scaffold in activity-based protein profiling.

5.4 Conclusion

Both the mechanistic study and the activity-based protein profiling performed in this Chapter give insight into the reactivity of the carbamate and the Michael acceptor present in the Brabantamide scaffold. The mechanistic studies provide insight in how an alcohol, an amine or a thiol react with methyl ester **2** and therefore enabled the design of new compounds with improved selectivity towards one or another nucleophile. Because we confirmed that methyl ester **2** undergoes Michael addition with thiols we decided to prepare vinyl sulfone based probes **VDG33** and **VDG30** to improve the reactivity towards the thiol of cysteine. The synthesized probes were indeed more potent towards the hyper reactive cysteine of RALDH1 in our activity-based protein profiling experiments. The ester based probe **PJD252** also showed an interesting profile as it did not label RALDH1 at all but displayed specificity towards a couple of serine hydrolases at 1 μM. These results confirm that the presence of both electrophiles does not prevent the synthesis of specific probes towards one residue. It can also be seen as an advantage towards a platform allowing access to various nucleophiles where ligand should be appended to promote affinity towards one or another target.

5.5 Experimental section

5.5.1 General biochemical procedures

Proteins. Recombinant Lp-PLA_2 (PAF-AH human, SRP3136 SIGMA) was purchased from Sigma-Aldrich. The protein was reconstituted in buffer (50 mM TRIS HCl pH 8.0, 150 mM NaCl) at 20 ng/μL in the presence of BSA (Bovin Serum Albumin) at 1 mg/mL and stored in 50 μL working aliquots at -20 °C. Recombinant Esterase from *Bacillus subtilis* (96667-10MG SIGMA) was purchased from Sigma-Aldrich. The protein was reconstituted in PBS (NaCl 137mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) at 100 ng/μL and stored in 100 μL working aliquots at -20 °C. Wild-type RALDH1 were obtained from the Sieber Lab. The protein was diluted to a concentration of 30 ng/μL in PBS and used as such.

Probes, reagents and material. Fp-rhodamine (ActivX™ TAMRA-FP Serine Hydrolase Probe, 88318) was purchased from Thermo Fisher Scientific and 10 μL working aliquots at 50 μM were prepared in DMSO and then stored at -20 °C. The probes and panel of derivatives were dissolved in DMSO and the stock solutions were stored at -20 °C.

Cell Culture conditions. A549 cells were grown in t75 culture flasks in DMEM supplemented

with 10% FBS (Fetal Bovine Serum), 1% L-Glutamine, and 1% Pen/Strep into an incubator at 37 °C and 5% CO_2 humidified air. At about 70-90% confluency, cells were detached from the flask by trypsin/EDTA treatment, pelleted and reseeded in 4 mL complete cell culture medium per t75 flask or washed two times with PBS and conserved at -80°C. The pellets were lysed using a NP40 lysis buffer [0.5 % NP40, Tris·HCl (10 mM), NaCl (150 mM), $MgCl_2$ (5 mM), pH 7.4] during 10 min over ice, the lysates were then spin down during 10 min at 10 000 rpm and the supernatant was collected and submitted to a Bradford protein assay to assess the protein concentration before subsequent dilution to 1 mg/mL. One t75 flask usually provided 200-250 µL at 1 mg/mL of protein.
Bacillus subtilis 168 cells were cultured in LB broth overnight. The cells were then diluted in LB to OD=0.1 and cultured 2-3 hours up to OD=0.4-0.5. The cells were then washed three times with PBS and lysed in PBS using lysozyme (1 mg/mL) together with sonication over ice. The lysates were then spin down during 10 min at 10 000 rpm and the supernatant was collected and submitted to a Bradford protein assay to assess the protein concentration. Lysates were then aliquoted, snap-frozen with liquid nitrogen and stored at -80 °C. Before each experiment the lysates were diluted to a concentration of 1 or 2 mg/mL of protein.

SDS-PAGE analysis. Laemmli type SDS-PAGE was performed according to standard literature procedures.[18] Gels were prepared using acrylamide-bis ready-to-use solution 40% (37.5:1) (Merck Millipore) and separated on a Mini-PROTEAN Tetra cell (Bio-Rad). Fluorescence scanning of SDS-PAGE gels was performed on a Typhoon 9500 FLA model (GE Healthcare) using the CY2 settings for BODIPY (blue laser excitation at 488 nm and emission filter at 520 nm) and CY3 settings for RHODAMINE. Coomassie staining was carried out with colloidal CBB G250 staining according to the manufactures protocol (Roti-Blue, Carl Roth). BioRad precision plus protein standards dual color was used as molecular weight marker.

5.5.2 Labeling experiments with recombinant proteins

SDS-PAGE labeling with recombinant Lp-PLA2.
1 µL of the probe **VDG33** or **PJD252** or **PJD253** (0.01 µM to 0.1 µM) was incubated with 9 µL of the Lp-PLA_2 solution (20 ng/µL stock solution) for 1 hour at 37 °C. SB (with DTT) was added and the proteins were resolved on a 15% SDS-PAGE. Fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning. (SB = sample buffer, DTT = dithiothreitol)

SDS-PAGE labeling with recombinant esterase from B.subtilis.
1 µL of the probe **VDG33** or **PJD252** or **PJD253** (0.1 µM to 1 µM) was incubated with 9 µL of the esterase (10 ng/µL stock solution) for 1 hour (enol carbamate) at 37 °C. SB (with DTT) was added and the proteins were resolved on a 12% SDS-PAGE. Fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

SDS-PAGE labeling with recombinant RALDH1.
1 µL of the probe **VDG33** or **PJD252** or **PJD253** (0.1 µM to 1 µM) was incubated with 9 µL of the raldh1 (30 ng/µL stock solution) for 1 hour (enol carbamate) at 37 °C. SB (with DTT) was added and the proteins were resolved on a 12% SDS-PAGE. Fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

5.5.3 Labeling experiments with lysates

SDS-PAGE labeling with A549 and B.subtilis lysates.

1 μL of probe **VDG33**, **PJD253** or **PJD252** was incubated one hour at 37 °C with 19 μL of lysates (1 mg/mL) and then 2 μL Fp-rhodamine (10 μM) was added and incubated during 30 minutes. SB (with DTT) was added and the proteins were resolved on a 12% SDS-PAGE. Fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

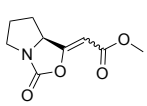
1 μL of two-step probe **VDG30**, **PJD243** and **PJD251** was incubated one hour at 37 °C with 19 μL of lysates (1 mg/mL) and then BODIPY-alkyne or BODIPY-azide was then clicked accordingly using the following conditions: BODIPY-N_3 or BODIPY-alkyne (100 μM), $CuSO_4{\cdot}5H_2O$ (100 μM), TABTA (500 μM) and sodium ascorbate (3 mM) for 2 hours. SB (with DTT) was then added and the proteins resolved on a 12% SDS-PAGE. Fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

SDS-PAGE competition experiments on A549 lysates.
1 μL of probe **19b-d** was incubated one hour at 37 °C with 19 μL of lysates (1 mg/mL) and then 2 μL Fp-rhodamine (10 μM) and 2 μL of **VDG33** were added and incubated during 30 minutes. SB (with DTT) was added and the proteins were resolved on a 12% SDS-PAGE and fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

1 μL of probe **disulfiram**, **iodoacetamide, VDG30** or **PJD2d** was incubated two hours at 37 °C with 19 μL of lysates (1 mg/mL) and then 1 μL of **VDG33** was added and incubated during 30 minutes. SB (with DTT) was added and the proteins were resolved on a 12% SDS-PAGE and fluorescence was visualized using a Typhoon scanner by in-gel fluorescence scanning.

5.5.4 NMR study - Synthetic procedure

General remarks. All reactions were performed using oven-dried glassware under an atmosphere of nitrogen (unless otherwise specified) using dry solvents. Reaction temperature refers to the temperature of the oil bath. Solvents were taken from a MBraun solvent purification system (SPS-800). All other reagents were purchased from Sigma Aldrich and Acros and used without further purification unless noted otherwise. Trimethylsilyl trifluoromethanesulfonate was stored under a nitrogen atmosphere in a dry Schlenk flask. TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using either ninhydrin stain (ninhydrin (1.5 g) and AcOH (3 mL) in *n*-butanol (100 mL)) or a $KMnO_4$ stain (K_2CO_3 (40 g), $KMnO_4$ (6 g), H_2O (600 mL) and 10% NaOH (5 mL)). Flash chromatography was performed using SiliCycle silica gel type SiliaFlash P60 (230 – 400 mesh) as obtained from Screening Devices.^{1}H- and ^{13}C-NMR spectra were recorded on a Varian AMX400 or a Varian 400-MR (400 and 100.59 MHz, respectively) using $CDCl_3$ as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: δ 7.26 for ^{1}H, δ 77.06 for ^{13}C). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, td = triple doublet, t = triplet, q = quartet, b = broad, m = multiplet), coupling constants *J* (Hz), and integration. High-resolution mass spectra (HRMS) were recorded on a Thermo Scientific LTQ Orbitrap XL.



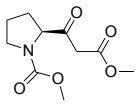
Methyl ester derivative 2. A solution of *N*-Boc Proline β-ketoester **1**. (523 mg, 1.93 mmol, 1 eq) in DCM (8 mL) was cooled to 0 ℃ before TMSOTf (700 μL, 3.87 mmol, 2 eq) was added. The reaction mixture was stirred until the starting material was completed consumed, then CDI (470 mg, 2.89 mmol, 1.5 eq) was added and stirred overnight. The reaction mixture was directly purified using flash

column chromatography with ethyl acetate: petroleum-ether (50 : 50) as eluent afforded the *Z* isomer of **2** (140 mg, 37% yield) as an oil and *E* isomer of **2** (72 mg, 19% yield). The spectral data were in accordance with literature.[12]

Z-isomer ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.12 (d, J = 1.5 Hz, 1H), 4.45 (ddd, J = 8.9, 6.9, 1.5 Hz, 1H), 3.71 (s, 3H), 3.70 – 3.58 (m, 1H), 3.35 – 3.21 (m, 1H), 2.28 – 2.17 (m, 1H), 2.17 – 1.98 (m, 2H), 1.68 (ddt, J = 12.1, 9.8, 8.5 Hz, 1H).

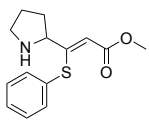
E-isomer ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.64 (s, 1H), 5.00 – 4.78 (m, 1H), 3.71 (s, 3H), 3.69 – 3.62 (m, 1H), 3.41 – 3.14 (m, 1H), 2.61 (s, 1H), 2.29 – 1.96 (m, 2H), 1.70 – 1.49 (m, 1H).

NMR-exp1 – MeOH + K_2CO_3



Enol cyclocarbamate **2** (20 mg, 0.10 mmol, 1 eq.) was dissolved in dry methanol (2 mL) and K_2CO_3 (13.8 mg, 0.10 mmol, 1 eq.) was added. After full consumption of the starting material, the reaction mixture was concentrated under reduced pressure and then purified by column chromatography using gradient of ethyl acetate in pentane (15-50%). The purification yielded methyl carbamate **3**. The NMR gives a mixture of rotamers. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.52 – 4.17 (m, 1H), 3.72 (s, 3H), 3.70 – 3.64 (m, 3H), 3.63 – 3.51 (m, 2H), 3.52 – 3.37 (m, 2H), 2.23 – 1.96 (m, 2H), 2.01 – 1.80 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 202.4, 202.1, 167.5, 167.2, 155.9, 154.9, 65.2, 52.7, 52.3, 47.3, 46.7, 46.5, 45.4, 29.7, 28.6, 24.5, 23.6. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{10}H_{16}NO_5$ = 230.10230, found: 230.10259; Calculated mass $[M + Na]^+$ $C_{10}H_{15}NNaO_5$ = 252.08479, found: 252.08460.

NMR-exp2 – Thiophenol + NaOH.

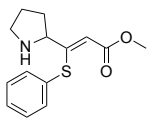


To a solution of 5 mL of 0.2 M NaOH was first added thiophenol (10.2 μL, 0.10 mmol, 1 eq.) and then enol cyclocarbamate **2** (20 mg, 0.10 mmol, 1 eq.) dissolved in 1 mL of acetonitrile. The reaction was stirred at room temperature overnight. After full consumption of the starting material, the reaction mixture was extracted with 2x 10 mL diethyl ether (organic layer A). The remaining water layer was neutralized using concentrated sulfuric acid and extracted using 2× 10 mL diethyl ether (organic layer B). Both organic layers were dried with Na_2SO_4 and concentrated under reduced pressure.

Organic layer A gave the E-isomer of enol-cyclocarbamate **2**.

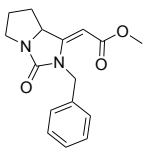
Organic layer B gave decarboxylated thiophenol adduct **4**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (d, J = 9.4 Hz, 2H), 7.38 (d, J = 6.2 Hz, 3H), 6.48 (s, 1H), 3.74 (s, 3H), 3.13 (m, 1H), 2.93 (m, 2H), 2.02 – 1.51 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.6, 136.2, 135.6, 130.4, 129.4, 129.3, 111.7, 60.6, 51.4, 46.4, 33.2, 30.3, 24.3. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{14}H_{17}NO_2S$ = 264.10528, found: 264.10586.

NMR-exp3 – Thiophenol + Et_3N.



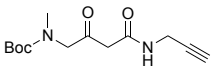
Enol cyclocarbamate **2** (20 mg, 0.10 mmol, 1 eq.) was dissolved in $CDCl_3$ (1 mL) and thiophenol (10.2 μL, 0.10 mmol, 1 eq.) was added. No conversion was observed by NMR after overnight incubation. Triethylamine (14 μL, 0.10 mmol, 1 eq.) was then added to the reaction mixture and NMR showed immediate consumption of the starting material. After full consumption of starting material, as judged by the disappearance of the typical double bound peak, the mixture was immediately purified using column chromatography with a gradient of methanol in DCM (0-5%) and concentrated under reduced pressure. The purification yielded the decarboxylated thiophenol adduct **4**. The ^{1}H NMR fully matched the one measured for NMR-exp2.

NMR-exp4 – Aniline + Benzylamine.

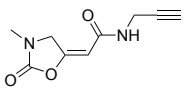


Enol cyclocarbamate **2** (20 mg, 0.10 mmol, 1 eq.) was dissolved in $CDCl_3$ (1 mL) and aniline (9.2 μL, 0.10 mmol, 1 eq.) was added. After overnight incubation, no conversion was observed by NMR. Benzylamine (11 μL, 0.10 mmol, 1 eq.) was then added and the NMR tube was heated at 40 ℃. Slow consumption of the starting material and concomitant formation of the benzylamine urea **5** was observed by NMR (typical ketone peak at 203 ppm was observed in ^{13}C NMR). After 4 hours the reaction was immediately purified using column chromatography (gradient of ethyl acetate in DCM: 0-5 %) and concentrated under reduced pressure. The purification yielded the ring-closed benzylamine adduct **6**. ^1H NMR (400 MHz, $CDCl_3$): δ 7.35 – 7.27 (m, 3H), 7.23 – 7.19 (m, 2H), 4.99 (s, 1H), 4.88 (ddd, J = 8.6, 6.5, 1.7 Hz, 1H), 4.66 (s, 2H), 3.70 (dt, J = 11.1, 7.8 Hz, 1H), 3.62 (s, 3H), 3.34 – 3.18 (m, 1H), 2.75 – 2.54 (m, 1H), 2.16 – 1.95 (m, 2H), 1.38 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 167.3, 160.9, 157.5, 135.2, 128.9, 127.9, 127.1, 88.7, 77.1, 63.3, 51.0, 45.6, 44.9, 30.4, 26.6. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{16}H_{19}N_2O_3$ = 287.13902, found: 287.13953; Calculated mass $[M + Na]^+$ $C_{16}H_{18}N_2NaO_3$ = 309.12151, found: 309.12144.

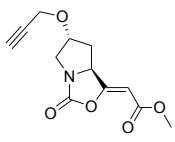
5.5.5 Probes and inhibitors - Synthetic procedure



***N*-Boc sarcosine β-ketoamide 9.** *N*-Boc sarcosine β-ketoester **8** prepared as described in Chapter 2 was converted into β-ketoamide **3** following the general procedure C described in Chapter 2. Briefly, 1,4-DABCO (660 mg, 5.83 mmol, 1.2 eq) was reacted with trimethylaluminium (5.83 mL, 11.66 mmol, 2.4 eq, 2 M in toluene) in toluene (10 mL) to produce DABAL *in situ*. Subsequently, propargylamine (376 μL, 5.83 mmol, 1.2 eq) in THF (7 mL) was added to activate the amine and finally the β-keto ester **8** was added (1.2 g, 4.89 mmol, 1 eq) in THF (3 mL). Flash chromatography using ethyl acetate: petroleum ether (1: 4) as eluent afforded 13 (704 mg, 55 % yield) as a yellow oil. Rf [silica, ethyl acetate: petroleum ether (1: 4)] = 0.25. ^1H NMR (400 MHz, $CDCl_3$): δ 4.13 – 4.01 (m, 2H), 3.41 (s, 2H), 2.92 (s, 3H), 2.23 (s, 1H), 1.57 (s, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 199.3, 154.7, 153.5, 80.8, 80.5, 65.9, 58.2, 48.3, 46.8, 46.7, 28.2, 24.5, 23.5. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{13}H_{21}N_2O_4$ = 269.14958, found: 269.15104.

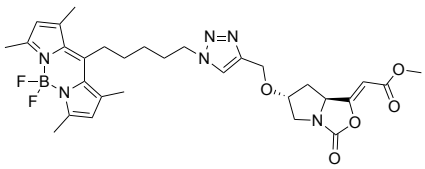


Methyl functionalized monocyclic derivative PJD251. This compound was prepared according to the general procedure D described in Chapter 2 by reacting the β-keto amide **9** (200 mg, 0.74 mmol, 1 eq), TMSOTf (280 μL, 1.48 mmol, 2 eq) and CDI (210 mg, 1.1 mmol, 1.5 eq) in DCM (3 mL). Flash chromatography using ethyl acetate: petroleum ether (1: 4) gave the E-isomer **PJD251** (15 mg, 10 % yield). Z-isomer could not be obtained pure. ^1H NMR (400 MHz, $CDCl_3$): δ 5.61 (s, 1H), 5.56 (t, J = 2.6 Hz, 1H), 4.67 (d, J = 2.5 Hz, 2H), 4.08 (dd, J = 5.4, 2.6 Hz, 2H), 2.99 (s, 3H), 2.25 (t, J = 2.5 Hz, 1H).^{13}C NMR (101 MHz, $CDCl_3$): δ 165.3, 159.4, 152.7, 105.2, 95.8, 79.4, 52.1, 30.6, 29.2. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_9H_{11}N_2O_3$ = 195.07642, found: 195.07744.

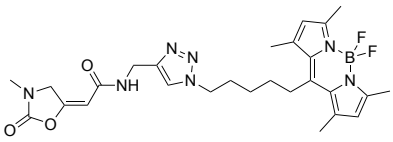


***trans*-4-propargyloxy-L-proline methyl ester derivative PJD243**. This compound was prepared according to the general procedure D described in Chapter 2 by reacting the appropriate β-keto ester **12** (50 mg, 0.23 mmol, 1 eq), TMSOTf (55 μL, 0.46 mmol, 2 eq) and CDI (42 mg, 0.35 mmol, 1.5 eq) in DCM (1 mL). Flash chromatography using ethyl acetate: pentane (1: 4) as eluent afforded **PJD243** (11.3 mg, 20 % yield) as a colorless oil. Rf [silica,

ethyl acetate: pentane (1: 4)] = 0.30. ; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.15 (d, J = 1.5 Hz, 1H), 4.69 (ddd, J = 10.7, 5.7, 1.2 Hz, 1H), 4.51 (t, J = 5.3 Hz, 1H), 4.18 (d, J = 2.4 Hz, 2H), 3.90 (dd, J = 12.7, 5.4 Hz, 1H), 3.74 (s, 3H), 3.36 (dd, J = 12.6, 1.3 Hz, 1H), 2.48 (t, J = 2.3 Hz, 1H), 2.40 (dd, J = 13.3, 6.0 Hz, 1H), 1.75 (ddd, J = 13.3, 10.6, 5.2 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.1, 159.8, 156.9, 94.8, 79.7, 77.2, 75.5, 62.3, 56.9, 52.7, 51.8, 38.0. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{12}H_{14}NO_5$ = 252.08665, found: 252.08648.



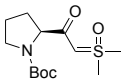
ester-BODIPY probe PJD252. This compound was prepared by reacting the appropriate alkyne probe **PJD243** (4 mg, 16 nmol, 1 eq) with BODIPY-azide (5.7 mg, 16 nmol, 1 eq) in the presence of $CuSO_4$ (32 μL of 100 mM stock solution in water, 3.2 nmol, 0.2 eq) and sodium ascorbate (48 μL of 100 mM stock solution in water, 4.8 nmol, 0.3 eq) in t-BuOH:MeOH:H_2O (1:1:1) (0.5 mL) for 18 hours. The reaction mixture was extracted with ethyl acetate and washed three times with water, subsequent flash chromatography using ethyl acetate: pentane (1: 1) as eluent afforded **PJD252** (4.62 mg, 48 % yield) as an orange powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.06 (s, 2H), 5.15 (s, 1H), 4.64 (bs, 2H), 4.47 (bs, 1H), 4.39 (bs, 1H), 4.02 – 3.84 (m, 1H), 3.75 (s, 3H), 3.36 (d, J = 12.4 Hz, 1H), 3.03 – 2.88 (m, 2H), 2.69 (t, J = 7.1 Hz, 1H), 2.51 (s, 6H), 2.39 (s, 6H), 2.09 – 1.92 (m, 2H), 1.82 – 1.49 (m, 7H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.1, 159.8, 157.0, 154.3, 143.9, 140.2, 131.5, 125.7, 121.9, 94.9, 80.2, 77.5, 77.2, 67.2, 62.4, 62.4, 52.9, 51.8, 39.0, 38.0, 31.4, 30.5, 28.2, 27.1, 23.7, 16.6, 14.6, 13.2. HRMS: (ESI+) Calculated mass $[M+NH_4]^+$ $C_{30}H_{37}BF_2N_6O_5NH_4$ = 628.32302, found: 628.32476; Calculated mass $[M-F]^+$ $C_{30}H_{37}BFN_6O_5$ = 591.29025, found: 591.29223.



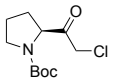
monocyclic-BODIPY probe PJD253. This compound was prepared by reacting the appropriate alkyne probe **PJD251** (3.1 mg, 16 nmol, 1 eq) with BODIPY-azide (5.7 mg, 16 nmol, 1 eq) in the presence of $CuSO_4$ (32 μL of 100 mM stock solution in water, 3.2 nmol, 0.2 eq) and sodium ascorbate (48 μL of 100 mM stock solution in water, 4.8 nmol, 0.3 eq) in t-BuOH:MeOH:H_2O (1:1:1) (0.5 mL) for 18 hours. The reaction mixture was extracted with ethyl acetate and washed three times with water, subsequent flash chromatography using ethyl acetate: pentane (1: 1) as eluent afforded **PJD253** (2.03 mg, 23 % yield) as an orange powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50 (s, 1H), 6.33 (t, J = 5.5 Hz, 1H), 6.05 (s, 2H), 5.60 (t, J = 2.5 Hz, 1H), 4.65 (d, J = 2.5 Hz, 2H), 4.56 (d, J = 5.3 Hz, 2H), 4.36 (t, J = 7.0 Hz, 2H), 2.98 (s, 3H), 2.97 – 2.92 (m, 2H), 2.51 (s, 6H), 2.38 (s, 6H), 1.97 (p, J = 7.1 Hz, 2H), 1.70 (m, 2H, + H2O), 1.50 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 158.6, 154.0, 145.3, 139.9, 133.0, 131.2, 117.5, 114.8, 96.0, 77.1, 51.8, 31.0, 30.3, 29.8, 27.8, 26.7, 25.8, 17.3, 16.3, 14.3. HRMS: (ESI+) Calculated mass $[M+Na]^+$ $C_{27}H_{34}BF_2N_7O_3Na$ = 576.26765, found: 576.26977; Calculated mass $[M-F]^+$ $C_{27}H_{34}BFN_7O_3$ = 534.28002, found: 534.28202.

***N*-Boc-L-proline-4-nitrophenyl ester 14.** The activated ester was prepared using a variation of the method described by Kovacs.[17] *N*-Boc-L-proline **13** (11.1 g, 51.7 mmol, 1 eq.) was dissolved in dichloromethane (100 mL). Then *N*,*N*'-dicyclohexylcarbodiimide (11.6 g 56.6 mmol 1.2 eq.), *p*-nitrophenol (7.1 g, 52 mmol, 1.1 eq.) and 4-dimethylaminopyridine (0.56 g, 4.6 mmol, 0.1 eq.) were added at 0 °C and the mixture was stirred until the starting material was fully consumed. The mixture was kept in the freezer at -21 °C for 20 minutes and the dicyclohexylurea (DCU) was subsequently filtered off. The mixture was washed twice with saturated $NaHCO_3$ solution.

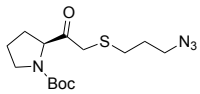
Then the crude mixture was extracted with DCM, dried with Na_2SO_4 and concentrated under reduced pressure to obtain **14** (13.2 g, 76% yield) as yellow oil. The resulting crude product contained <10% nitrophenol, and was used for the next step without further purification.



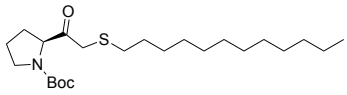
N*-boc-L-proline-β-keto sulphur ylide 15.** Adapted from a procedure reported by Wang ***et al.[16] To a solution of trimethylsulfoxonium iodide (9 g, 41 mmol, 2.2 eq.) in THF (27 mL) was added potassium *tert*-butoxide (1M in THF, 41 mL, 41 mmol, 2 eq.) at room temperature. The reaction mixture was refluxed for 4 hours and then cooled to 0 °C before *N*-Boc-L-proline-4-nitrophenol ester **14** (6.2 g, 18.64 mmol, 1 eq.) in THF (9.3 mL) was added dropwise. The reaction mixture was stirred at 0 °C until the starting material was fully consumed. The reaction was quenched by adding ethyl acetate (27 mL) and subsequently filtered over celite. The crude mixture was finally concentrated under reduced pressure yielding **15** (4.7 g, 87% yield) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.48 (d, J = 11.0 Hz, 1H), 4.24 – 3.98 (m, 1H), 3.51 – 3.42 (m, 1H), 3.38 (d, J = 7.4 Hz, 6H), 2.20 – 2.07 (m, 1H), 2.07 – 1.71 (m, 3H), 1.44 (bs, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 191.3, 154.6, 79.5, 67.5, 67.0, 64.4, 63.8, 47.2, 47.2, 46.8, 42.5, 31.7, 30.5, 28.7, 24.4, 23.7. IR νmax/cm−1: 2976, 1685, 1567, 1392, 1366, 1168, 1118, 1030, 920, 893, 857, 771 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{13}H_{24}NO_4S$ = 290.14261, found: 290.14233.



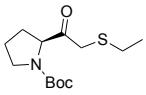
***N*-Boc-L-proline-α-chloroketone 16.** Adapted from the procedure reported by Wang *et al*.[16] A solution of *N*-Boc-L-proline-β-keto sulphur ylide **15** (0.25 g, 0.87 mmol, 1 eq.) in THF (6 mL) was cooled to 0 °C before LiCl (55 mg, 1.3 mmol, 1.5 eq.) and camphorsulfonic acid (0.19 ml, 0.82 mmol, 0.95 eq.) were added. The temperature was slowly raised to 70 °C and the mixture was stirred until the starting material was fully consumed. The reaction mixture was directly filtered over silica and subsequently concentrated under reduced pressure. The crude mixture was dissolved in ethyl acetate (1 mL) and filtered again over silica and subsequently concentrated under reduced pressure to obtain **16** (0.57g, 69% yield) as light brown oil. The spectral data were in accordance with previously published data.[19] ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.63 – 4.39 (m, 1H), 4.41 – 4.08 (m, 2H), 3.62 – 3.37 (m, 2H), 2.32 – 2.08 (m, 1H), 2.04 – 1.82 (m, 3H), 1.43 (d, J = 14.6 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 202.4, 115.8, 105.2, 80.5, 63.5, 62.9, 47.3, 47.1, 46.2, 30.6, 29.5, 28.5, 24.8, 24.0. IR ν_{max}/cm^{-1}: 2978, 1693, 1650, 1393, 1366, 1160, 926, 855, 775 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{11}H_{19}Cl_1N_1O_3$ = 248.10535, found: 248.10480.



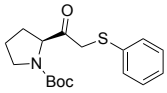
***N*-Boc-L-proline-(3-azidopropyl)sulphide 17a.** To a solution of *S*-(3-azidopropyl)thioacetate (0.37 mL, 2.67 mmol, 1.1 eq.) in 1:1 MeOH:H_2O (0.64 mL), K_2CO_3 (0.67 g, 4.86 mmol, 2 eq.) and *N*-Boc-L-proline-α-chloroketone **16** (0.6 g, 2.43 mmol, 1 eq.) were added at room temperature. The reaction mixture was stirred until complete consumption of the starting material was observed and then the reaction mixture was poured directly in ethyl acetate (12.4 mL). The organic layer was separated, dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography using ethyl acetate : petroleum-ether 45-60 °C (25 : 75) as eluent yielded **17a** (0.65 g, 82% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.60 – 4.29 (m, 1H), 3.60 – 3.44 (m, 1H), 3.43 – 3.35 (m, 2H), 3.33 (d, J = 5.4 Hz, 1H), 2.64 – 2.54 (m, 2H), 2.34 – 2.07 (m, 2H), 2.04 – 1.75 (m, 6H), 1.43 (d, J = 10.9 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 204.8, 154.7, 153.7, 80.5, 79.9, 77.3, 76.7, 65.8, 64.1, 63.3, 50.0, 49.9, 47.0, 38.7, 37.7, 31.1, 29.8, 29.2, 29.0, 28.4, 28.1, 24.6, 23.8. IR νmax/cm^{-1}: 2975, 2096, 1688, 1392, 1366, 1160, 1117, 903, 771, 557, 532 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{14}H_{25}N_4O_3S$ = 329.16474, found: 329.16419.



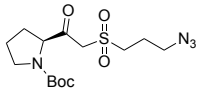
***N*-Boc-L-proline-(dodecane)sulfide 17b.** To a solution of dodecanethiol (0.31 mL, 1.78 mmol, 1.1 eq) in 1:1 MeOH: H_2O (8.5 mL), K_2CO_3 (0.25 g, 1.78 mmol, 1.1 eq.) and *N*-Boc-L-proline-α-chloroketone **16** (0.4 g, 1.62 mmol, 1 eq.) were added at room temperature. The reaction mixture was stirred until complete consumption of the starting material was observed and then the reaction mixture was poured directly in ethyl acetate (25 mL). The organic layer was separated, dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography using ethyl acetate: pentane (15 : 85) as eluent yielded **17b** (0.36 g, 54 % yield) as off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.61 – 4.32 (m, 1H), 3.59 – 3.42 (m, 2H), 3.39 – 3.32 (m, 2H), 2.54 – 2.42 (m, 2H), 2.05 – 1.89 (m, 2H), 1.89 – 1.79 (m, 1H), 1.64 – 1.51 (m, 3H), 1.43 (d, J = 11.0 Hz, 9H), 1.25 (s, 18H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 205.9, 80.9, 64.4, 63.7, 47.3, 39.6, 38.6, 32.8, 32.8, 32.4, 32.1, 31.7, 31.4, 30.6, 30.4, 30.1, 29.7, 29.2, 25.2, 24.3, 23.2, 14.6. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{23}H_{44}NO_3S$ = 414.30364, found: 414.30311; Calculated mass $[M + Na]^+$ $C_{23}H_{43}NO_3SNa$ = 436.28613, found: 436.28508.



***N*-Boc-L-proline-(ethane)sulfide 17c.** To a solution of ethanethiol (0.093 mL, 1.78 mmol, 1.1 eq) in 1:1 MeOH:H_2O (8.3 mL) were added K_2CO_3 (0.25 g, 1.78 mmol, 1.1 eq) and *N*-Boc-L-proline-α-chloroketone **16** (0.4 g, 1.62 mmol, 1 eq) at room temperature. The reaction mixture was stirred until complete consumption of the starting material was observed and then the reaction mixture was poured directly in ethyl acetate (25 mL). The organic layer was separated, dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography using ethyl acetate: pentane (15: 85) as eluent yielded **17c** (0.32 g, 73 % yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.55 – 4.34 (m, 1H), 3.61 – 3.47 (m, 2H), 3.43 – 3.28 (m, 2H), 2.59 – 2.46 (m, 2H), 2.35 – 2.10 (m, 1H), 2.01 – 1.82 (m, 3H), 1.42 (s, 9H), 1.32 – 1.19 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 205.5, 80.9, 80.4, 64.4, 63.7, 47.3, 39.1, 38.1, 31.7, 30.5, 28.9, 28.8, 26.7, 25.1, 24.3, 14.5. HRMS: (ESI+) Calculated mass $C_{13}H_{23}NO_3SNa$ = 296.12909, found: 296.12908.

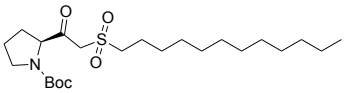


***N*-Boc-L-proline-(benzene)sulfide 17d**. To a solution of thiolphenol (0.067 mL, 0.57 mmol, 1.1 eq) in 1:1 MeOH:H_2O (3.1 mL), K_2CO_3 (79 mg, 0.57 mmol, 1.1 eq) and *N*-Boc-L-proline-α-chloroketone **16** (0.13 g, 0.52 mmol, 1 eq) were added at room temperature. The reaction mixture was stirred until complete consumption of the starting material was observed and then the reaction mixture was poured directly in ethyl acetate (10 mL). The organic layer was separated, dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography using ethyl acetate: pentane (10: 90) as eluent yielded **17d** (0.12 g, 71 % yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 – 7.10 (m, 5H), 4.66 – 4.34 (m, 1H), 3.89 (s, 1H), 3.81 (d, J = 4.8 Hz, 1H), 3.64 – 3.32 (m, 2H), 2.26 – 1.98 (m, 2H), 1.91 – 1.74 (m, 2H), 1.48 – 1.42 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 205.5, 155.2, 135.7, 130.1, 129.5, 127.4, 126.9, 81.0, 80.5, 77.2, 64.5, 63.7, 47.5, 42.5, 42.0, 31.1, 30.0, 28.8, 25.2, 24.3. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{17}H_{24}NO_3S$ = 322.14714, found: 322.14698; Calculated mass $[M + Na]^+$ $C_{17}H_{23}NO_3SNa$ = 344.12909, found: 344.12903.

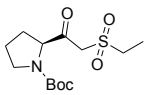


***N*-Boc-L-proline-(3-azidopropyl)sulfone 18a.** A solution of **17a** (150 mg, 0.46 mmol, 1 eq) in DCM (1.05 mL) was treated at room temperature with *meta*-chloroperoxybenzoic acid (*m*CPBA) (78.9mg, 1.4mmol, 3 eq) and was stirred at room temperature until the start material was fully consumed. The reaction mixture was washed four times with sat. $NaHCO_3$,

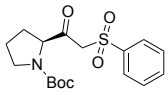
dried over Na_2SO_4 and concentrated under reduced pressure to obtain **18a** (0.12 g, 72% yield) as colorless oil. The material contained <10% of *m*CPBA and was used as such in the next step. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.44 – 3.91 (m, 3H), 3.58 – 3.41 (m, 4H), 3.36 (t, J = 6.5 Hz, 2H), 2.23 – 2.05 (m, 4H), 2.03 – 1.83 (m, 2H), 1.46 – 1.42 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 199.5, 155.1, 80.9, 66.8, 66.1, 59.8, 59.0, 51.3, 49.8, 47.2, 28.5, 28.2, 24.8, 23.9, 21.9. IR ν_{max}/cm^{-1}: 2977, 2099, 1682, 1393, 1366, 1316, 1257, 1160, 1129, 1045, 1022, 899, 856, 799, 772, 506, 440 cm^{-1}. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{14}H_{25}N_4O_5S$ = 361.15457, found: 361.15402.



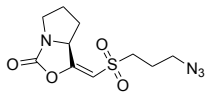
***N*-Boc-L-proline-(dodecane)sulfone 18b.** A solution of **17b** (340 mg, 0.82 mmol, 1 eq.) in DCM (2.3 mL) was treated at room temperature with *m*CPBA (425 mg, 2.5 mmol, 3 eq.) and was stirred at room temperature until the start material was fully consumed. The reaction mixture was dissolved in ethyl acetate (10 mL), washed four times with sat. $NaHCO_3$, dried over Na_2SO_4 and concentrated under reduced pressure to obtain **18b** (0.35 g, 96% yield) as a off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.44 – 4.31 (m, 1H), 4.25 – 3.92 (m, 2H), 3.47 (t, J = 6.9 Hz, 2H), 3.28 – 3.20 (m, 2H), 2.18 – 2.07 (m, 2H), 2.02 – 1.88 (m, 2H), 1.88 – 1.78 (m, 2H), 1.44 (s, 9H), 1.26 (s, 18H), 0.88 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 199.5, 81.1, 66.0, 59.3, 54.0, 47.0, 31.9, 30.6, 29.6, 29.3, 28.6, 28.4, 28.3, 28.0, 25.2, 25.1, 24.6, 22.7, 21.8, 21.6, 21.2, 14.1. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{23}H_{44}NO_5S$ = 446.29402, found: 446.29271; Calculated mass $[M + Na]^+$ $C_{23}H_{43}NO_5SNa$ = 468.27542, found: 468.27483.



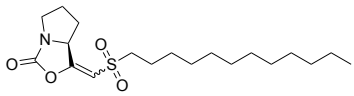
***N*-Boc-L-proline-(ethane)sulfone 18c.** A solution of **17c** (290 mg, 1.06 mmol, 1 eq.) in DCM (2.1 mL) was treated at room temperature with *m*CPBA (550 mg, 3.2 mmol, 3 eq.) and was stirred at room temperature until the start material was fully consumed. The reaction mixture was dissolved in ethyl acetate (5 mL), washed four times with sat. $NaHCO_3$, dried over Na_2SO_4 and concentrated under reduced pressure to obtain **18c** (0.28 g, 87 % yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.39 – 4.28 (m, 1H), 4.26 – 4.01 (m, 2H), 3.46 (t, J = 6.9 Hz, 2H), 3.36 – 3.18 (m, 2H), 2.16 – 2.09 (m, 2H), 2.00 – 1.84 (m, 2H), 1.44 (s, 9H), 1.41 (t, J = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 199.3, 154.7, 104.8, 80.8, 80.5, 77.2, 66.4, 65.9, 58.2, 57.5, 48.3, 46.8, 46.7, 28.8, 28.2, 27.8, 24.5, 23.5, 16.4. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{13}H_{24}NO_5S$ = 306.13697, found: 306.13701; Calculated mass $[M + Na]^+$ $C_{17}H_{23}NO_5SNa$ = 328.11891, found: 328.11905.



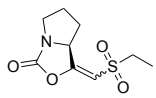
***N*-Boc-L-proline-(benzene)sulfone 18d.** A solution of **17d** (92 mg, 0.29 mmol, 1 eq.) in DCM (0.6 ml) was treated at room temperature with *m*CPBA (150 mg, 0.86 mmol, 3 eq.) and was stirred at room temperature until the start material was fully consumed. The reaction mixture was dissolved in ethyl acetate (5 mL), washed four times with sat. $NaHCO_3$, dried over Na_2SO_4 and concentrated under reduced pressure to obtain **18d** (88 mg, 87% yield) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 – 7.89 (m, 2H), 7.67 (t, J = 8.7 Hz, 1H), 7.58 (m, 2H), 4.49 – 4.33 (m, 2H), 4.31 – 4.18 (m, 1H), 3.56 – 3.35 (m, 2H), 2.28 – 2.08 (m, 2H), 1.98 – 1.81 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 198.3, 133.7, 130.7, 130.2, 129.6, 128.9, 128.3, 81.4, 80.9, 77.2, 66.6, 66.0, 64.3, 63.0, 47.5, 47.2, 29.7, 28.8, 28.7, 25.0, 24.1. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{17}H_{24}NO_5S$ = 354.13697, found: 354.13678; Calculated mass $[M + Na]^+$ $C_{17}H_{23}NO_5SNa$ = 376.11891, found: 376.11870.



(3-azidopropyl)sulfone carbamate VDG30. A solution of **18a** (56 mg, 0.15 mmol, 1 eq.) in DCM (1 mL) was cooled to 0 °C before TMSOTf (56 μL, 0.31 mmol, 2 eq.) was added. The reaction mixture was stirred until the starting material was completed consumed, then CDI (37.6 mg, 0.23 mmol, 1.5 eq.) was added and stirred overnight. The reaction mixture was directly purified using flash column chromatography with ethyl acetate : petroleum-ether (50 : 50) as eluent afforded the *E* isomer of **VDG30** (20.8 mg, 47% yield) as a yellow oil. *E*-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.01 (s, 1H), 5.11 – 4.84 (m, 1H), 3.68 (dt, J = 11.6, 7.9 Hz, 1H), 3.51 (t, J = 6.4 Hz, 2H), 3.31 (ddd, J = 12.2, 9.3, 4.5 Hz, 1H), 3.13 (t, J = 7.6 Hz, 2H), 2.76 – 2.50 (m, 1H), 2.26 – 1.99 (m, 3H), 1.83 – 1.63 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.7, 155.5, 103.1, 63.4, 53.8, 49.6, 46.0, 31.9, 26.3, 22.6. IR ν_{max}/cm^{-1}: 3060, 2928, 2101, 1798, 1660, 1370, 1314, 1125, 1030, 985, 814, 758, 663, 525 cm^{-1}. HRMS: (ESI+) Calculated mass $[M+H]^+$ $C_{10}H_{15}N_4O_4S$ = 287.08085 found: 287.08087; Calculated mass $[M+NH_4]^+$ $C_{10}H_{14}N_4O_4SNH_4$ = 304.10740, found: 304.10766.



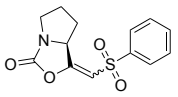
dodecane-sulfone carbamate 19b. A solution of **18b** (194 mg, 0.43 mmol, 1 eq.) in DCM (2 mL) was cooled to 0 °C before TMSOTf (155 μL, 0.86 mmol, 2 eq.) was added. The reaction mixture was stirred until the starting material was completed consumed, then CDI (100 mg, 0.65 mmol, 1.5 eq.) was added and stirred overnight. The reaction mixture was directly purified using flash column chromatography with ethyl acetate : pentane (4: 6) as eluent afforded *E*-**19b** (17.07 mg, 11% yield) as a yellow oil and *Z*-**19b** (28.4 mg, 17% yield). *E*-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.98 (s, 1H), 4.91 (dd, J = 9.4, 6.8 Hz, 1H), 3.75 – 3.60 (m, 1H), 3.35 – 3.24 (m, 1H), 3.09 – 2.95 (m, 2H), 2.71 – 2.53 (m, 1H), 2.19 – 2.05 (m, 2H), 1.85 – 1.74 (m, 4H), 1.53 – 1.38 (m, 2H), 1.25 (s, 16H), 0.93 – 0.83 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.9, 155.8, 103.3, 77.2, 63.4, 56.8, 46.0, 32.0, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 28.4, 25.2, 23.6, 22.8, 14.3. The product contains *m*CPBA as an impurity (around 10%). *Z*-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.64 (d, J = 1.6 Hz, 1H), 4.51 (ddd, J = 9.0, 6.9, 1.7 Hz, 1H), 3.68 (dt, J = 11.4, 7.8 Hz, 1H), 3.33 (ddd, J = 11.3, 8.7, 4.3 Hz, 1H), 3.20 – 3.14 (m, 2H), 2.32 – 2.02 (m, 3H), 1.85 – 1.67 (m, 3H), 1.42 (p, J = 7.2 Hz, 2H), 1.25 (broad s, 16H), 0.87 (t, J = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.2, 155.6, 103.8, 77.2, 63.4, 55.8, 46.3, 32.0, 31.3, 29.7, 29.4, 29.5, 29.4, 29.2, 28.5, 26.6, 22.8, 22.4, 14.3. HRMS: (ESI+) Calculated mass $[M+NH_4]^+$ $C_{19}H_{33}NO_4SNH_4$ = 389.24740, found: 389.24918.



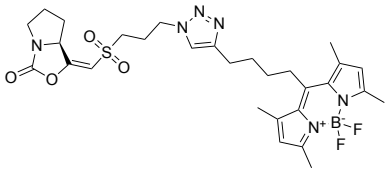
ethane-sulfone carbamate 19c. A solution of **18c** (225 mg, 0.74 mmol, 1 eq.) in DCM (1 mL) was cooled to 0 °C before TMSOTf (261 μL, 1.48 mmol, 2 eq.) was added. The reaction mixture was stirred until the starting material was completed consumed, then CDI (171 mg, 1.11 mmol, 1.5 eq.) was added and stirred overnight. The reaction mixture was directly purified using flash column chromatography with ethyl acetate : pentane (7: 3) as eluent afforded *E*-**19c** (10 mg, 6% yield) as a yellow oil and *Z*-**19c** (30.5 mg, 18% yield). *E*-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.97 (d, J = 2.0 Hz, 1H), 4.92 (ddd, J = 9.6, 6.6, 2.0 Hz, 1H), 3.68 (dt, J = 11.4, 8.0 Hz, 1H), 3.30 (ddd, J = 11.4, 9.0, 4.3 Hz, 1H), 3.06 (q, J = 7.4 Hz, 2H), 2.62 (dtd, J = 13.1, 6.9, 2.9 Hz, 1H), 2.18 – 2.00 (m, 2H), 1.79 – 1.64 (m, 1H), 1.39 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.3, 155.8, 102.5, 77.2, 63.4, 51.1, 46.0, 32.1, 26.3. HRMS: (ESI+) Calculated mass $[M+NH_4]^+$ $C_9H_{13}NO_4SNH_4$ = 249.09035, found: 249.09175.

Z-isomer: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.63 (bs, 1H), 4.52 (ddt, J = 9.5, 7.0, 1.4 Hz, 1H), 3.67 (ddd, J = 11.4, 8.2, 7.0 Hz, 1H), 3.33 (ddd, J = 12.1, 9.2, 4.3 Hz, 1H), 3.20 (q, J = 7.4 Hz, 2H), 2.32 –

2.04 (m, 3H), 1.86 – 1.68 (m, 1H), 1.37 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 159.6, 155.5, 102.9, 77.2, 63.4, 50.1, 46.3, 31.3, 26.6. HRMS: (ESI+) Calculated mass $[M+NH_4]^+$ $C_9H_{13}NO_4SNH_4$ = 249.09035, found: 249.09192.



benzene-sulfone carbamate 19d. A solution of **18d** (53 mg, 0.15 mmol, 1 eq.) in DCM (1 mL) was cooled to 0 °C before TMSOTf (53 µL, 0.30 mmol, 2 eq.) was added. The reaction mixture was stirred until the starting material was completed consumed, then CDI (35.5 mg, 0.23 mmol, 1.5 eq.) was added and stirred overnight. The reaction mixture was directly purified using flash column chromatography with ethyl acetate : pentane (5: 5) as eluent afforded *E*-**19d** (6.27 mg, 15% yield) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 – 7.95 (m, 1H), 7.89 (m, 2H), 7.57 (m, 2H), 6.07 (t, J = 1.7 Hz, 1H), 5.05 (ddt, J = 9.9, 6.6, 1.7 Hz, 1H), 3.75 – 3.62 (m, 1H), 3.40 – 3.26 (m, 1H), 2.82 – 2.70 (m, 2H), 2.22 – 2.08 (m, 3H), 1.89 – 1.69 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 162.1, 141.6, 134.5, 133.7, 129.5, 127.9, 126.9, 106.3, 63.3, 45.9, 31.8, 26.2. The product contains *m*CPBA as an impurity (around 10%).



BODIPY-sulfone carbamate VDG33. To a solution of carbamate **VDG30** (5 mg, 0.017 mmol, 1 eq.) and BODIPY-alkyne (5.7, 17 µmol, 1 eq.) in *tert*-butanol : MeOH : H_2O (0.1 : 0.2 : 0.1 mL) were added sodium ascorbate (52 µL, 5.2 µmol, 30 mol%) and $CuSO_4$ (35 µL, 3.5 µmol, 20 mol%). (Sodium ascorbate was added from a 100 mM stock solution in Milli Q water and $CuSO_4$ was added from a 100 mM stock solution in Milli Q water.) When the reaction reached completion after 18 hours, ethyl acetate was added and the crude mixture was washed twice with water. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure. Purification by silica gel flash column chromatography using ethyl acetate as eluent yielded **VDG33** (2.2 mg, 21% yield) as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33 (s, 1H), 6.05 (s, 2H), 5.97 (s, 1H), 4.96 – 4.83 (m, 2H), 4.52 (t, J = 6.6 Hz, 2H), 3.68 (dt, J = 11.5, 7.9 Hz, 2H), 3.30 (ddd, J = 11.1, 8.8, 4.2 Hz, 2H), 3.06 (t, J = 7.4 Hz, 2H), 3.00 (t, J = 8.4 Hz, 2H), 2.87 – 2.75 (m, 1H), 2.51 (s, 6H), 2.45 (t, J = 6.9 Hz, 2H), 2.39 (s, 6H), 2.23 – 2.03 (m, 2H), 2.00 – 1.88 (m, 2H), 1.75 – 1.63 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 164.9, 154.1, 146.1, 140.4, 131.6, 121.8, 103.0, 63.5, 53.4, 48.1, 46.0, 31.9, 31.6, 29.9, 29.6, 28.2, 26.3, 25.5, 23.5, 16.6, 14.6. HRMS: (ESI+) Calculated mass $[M + H]^+$ $C_{29}H_{38}BF_2N_6O_4S$ = 615.27364, found: 615.27236; Calculated mass $[M + Na]^+$ $C_{29}H_{37}BF_2N_6O_4SNa$= 637.25558, found: 637.25432.

5.6 References

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